Estimating Losses of Dry Matter from Wetted Alfalfa–Orchardgrass Mixtures Using Cell Wall Components as Internal Markers

W. K. Coblentz* and W. E. Jokela

ABSTRACT

Methods previously used to measure recoveries of dry matter (DM) from forages following natural or simulated rainfall often have relied on simple gravimetric techniques, which yielded inconclusive estimates of DM recovery. Our objective was to evaluate insoluble cell-wall constituents as internal markers for estimating recoveries of DM from alfalfa (*Medicago sativa* L.) or alfalfa–orchardgrass (*Dactylis glomerata* L.) mixtures subjected to simulated rainfall. Forage mixtures consisted of 1000, 750, or 500 g kg^{-1} alfalfa, with the balance comprised of orchardgrass. Regardless of the forage mixture, concentrations of all cell-wall constituents increased ($P \leq 0.053$) in response to wetting (0, 70, 140, 280, 420, 560, or 840 mm at 70 mm h^{-1}) under a rainfall simulator. Following treatment, recovery of all cell-wall constituents was high (≥901 g kg^{-1}). Generally, losses were smallest for neutral-detergent fiber (NDF) markers regardless of analysis method (with alpha-amylase, sodium sulfite, neither, or both), acid-detergent fiber (ADF), and cellulose and largest for hemicellulose and lignin. Linear regressions of recoveries of DM by internal markers on values determined gravimetrically were good ($r^2 \geq 0.775$) when NDF was used to estimate recovery. In all these cases, neither slopes ($P \geq 0.103$) nor intercepts ($P \geq 0.083$) differed from one and zero, respectively. Fiber components appear to be suitable internal markers for measuring recoveries of DM following wetting, but this approach depends on complete recovery of shattered leaf particles before conducting laboratory analyses.

USDA-ARS, U.S. Dairy Forage Research Center, 8396 Yellowstone Dr., Marshfield, WI 54449. Mention of trade names or commercial products is solely for the purpose of providing specific information about scientific procedures and/or subsequent evaluation of results and does not imply any recommendation or endorsement by the USDA. Received 4 Jan. 2008. *Corresponding author (wayne.coblentz@ars.usda.gov).

Abbreviations: ADF, acid-detergent fiber; ADF*as*, acid-detergent fiber determined sequentially following initial extraction in neutral detergent containing both α-amylase and sodium sulfite; ALF, proportion of alfalfa, in g kg^{-1}; CELL*as*, cellulose determined sequentially following initial extraction in neutral detergent containing both α-amylase and sodium sulfite; DM, dry matter; HEMI*as*, hemicellulose determined sequentially following initial extraction in neutral detergent containing both α-amylase and sodium sulfite; LIG*as*, acid-detergent lignin determined sequentially following initial extraction in neutral detergent containing both α-amylase and sodium sulfite; NDF, neutral-detergent fiber without additives; NDF*a*, neutral-detergent fiber with heat-stable α-amylase; NDF*as*, neutral-detergent fiber with heat-stable α-amylase and sodium sulfite; NDF*s*, neutral-detergent fiber with sodium sulfite.

The frustrations of hay producers attempting to produce high-quality hay during periods of unstable or inclement weather are widely known. Rain damage to wilting forage crops occurs frequently, and the negative effects of rain damage on wilting forage crops have been outlined in several research reports (Collins, 1982; Rotz and Abrams, 1988; Smith and Brown, 1994; Scarbrough et al., 2004, 2005). Generally, soluble cell components are leached from forage tissues (Sundberg and Thylén, 1994), and the primary leachates are nonstructural carbohydrates (Collins, 1982). This process accounts, directly, for losses of dry matter (DM) from the hay crop and, indirectly, for increased

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677 S. Segoe Rd., Madison, WI 53711 USA

concentrations of cell-wall components (Collins, 1982, 1983; Rotz et al., 1991; Scarbrough et al., 2005).

Methodologies used to estimate losses of DM in experiments with rain-damaged forages have often relied on simple gravimetric techniques, but these approaches have proven to be problematic. In some reports (Rotz et al., 1991; Rotz and Abrams, 1988), mowed forages were weighed into wire-mesh trays either before natural rainfall events or before artificial rainfall was applied via simulation techniques. Both reports noted numerous problems associated with these methods, including estimates of DM loss from wilting alfalfa (*Medicago sativa* L.) that were less than zero, thereby suggesting falsely that DM was actually produced as a result of rainfall events. Similarly, Gordon et al. (1969) reported highly variable, and sometimes negative, estimates of DM loss from rain-damaged alfalfa and orchardgrass (*Dactylis glomerata* L.) forages.

Rotz et al. (1991) suggested that these difficulties are associated with small fluctuations in estimates of the initial DM concentration of each experimental forage. In part, this error may be associated with the somewhat dubious assumption that all forage in the experimental basket, tray, or windrow is uniform and that a single estimate of DM concentration can represent adequately the forage before application of simulated or natural rainfall. Regardless of the source of these errors, alternative methodologies based on more precise methods are needed to provide better estimates of rainfall-induced losses of DM.

An alternative technique is based on the principle that most cell-wall components are insoluble in water (Van Soest, 1982). Although concentrations of cell-wall constituents generally increase in response to rain damage, these responses are associated with decreased concentrations of cell-soluble constituents (particularly sugars) that are leached from the forage (Collins, 1982); in theory, the actual pool of cell-wall components should remain relatively inert and largely unaffected. Therefore, insoluble cell-wall components could be excellent candidates for use as internal markers to estimate losses of DM from rain-damaged forages (Fonnesbeck et al., 1986). Previously, Salo and Virtanen (1983) applied this approach, calculating losses of DM ranging from 120 to 290 g kg^{-1} in rain-damaged, cool-season grass hays using lignin as an internal marker. Most recently, Scarbrough et al. (2004) conducted a marker validation study and found that losses of DM from rain-damaged orchardgrass and bermudagrass [*Cynodon dactylon* (L.) Pers.] hays were best estimated using neutral-detergent fiber without additives (NDF) as an internal marker. This is especially convenient because NDF is included in virtually all forage-analysis packages, and it requires no additional equipment or facilities beyond those used commonly in most forage-analysis laboratories.

While estimation of DM loss or recovery with internal markers may be superior to simple gravimetric techniques, rigorous evaluation of internal markers for estimating DM recoveries from alfalfa and alfalfa–grass mixtures has not been attempted. While the work of Scarbrough et al. (2004) validated an internal marker approach based on cell-wall components within perennial cool- and warm-season grasses, it remains unclear whether this research technique is equally appropriate for legumes. Several physical and/or compositional differences between grasses and legumes potentially could confound or invalidate this technique. An incomplete list of these differences exhibited by legumes in contrast to grasses includes: (i) lower concentrations of NDF and hemicellulose (Van Soest, 1982); (ii) increased lignification, especially within stem tissues (Coblentz et al., 1998); (iii) reduced association of N with NDF, and presumably the cell wall (Ogden et al., 2006); (iv) increased concentrations of pectin and starch (Van Soest, 1982); and (v) vast increases in leaf fragility relative to stem tissue. Therefore, the objective of this study was to validate insoluble cell-wall constituents as internal markers to estimate recoveries of DM from alfalfa or alfalfa–orchardgrass mixtures damaged by simulated rainfall.

EXPERIMENTAL PROCEDURES

Experimental Forages

Forages for this marker-validation study were obtained from the third cutting of an 8.2-ha field comprised of 'Phabulous II' alfalfa and 'Extend' orchardgrass that were established on 14 April 2004 near Stratford, WI (44°7′ N, 90°1′ W), on a Loyal silt loam soil (fine-loamy, mixed, Oxyaquic Glossudalfs). Soil fertility tests conducted during the fall of 2006 indicated that pH = 6.9, P = 114 mg kg^{-1}, K = 246 mg kg^{-1}, and organic matter = 37 g kg^{-1}. On a dry-weight basis, the forage mixture consisted of 760 g kg^{-1} alfalfa and 224 g kg^{-1} orchardgrass at the time it was mowed and conditioned on 23 July 2007 at 1030 h. After mowing, forages were gathered in bulk at approximately 1300 h and removed to a building for processing. Forage samples were immediately hand separated by species, and forage-particle length was reduced to approximately 5 cm with a manually operated paper cutter. Any weeds or other forage species, such as volunteer clovers or other grasses, were discarded during the hand-separation process. Forages were then weighed into 18 × 30-cm custom-made Dacron bags (5.8 ± 0.06 g; 53-μm pore size; ANKOM Technology, Fairport, NY) to create three different proportions of alfalfa (1000, 750, or 500 g kg^{-1}), designated as 1000ALF, 750ALF, and 500ALF, respectively. In each case, the balance of the forage in each bag was comprised of orchardgrass (0, 250, or 500 g kg^{-1}, respectively). Proportions of each forage were determined solely on the basis of wet (as is) forage weight. After bags were filled, the average total weight of wet forage in each bag was 101.1 ± 1.15 g (26.0 ± 0.79 g DM). It should be noted that Dacron bags of this type are designed specifically for evaluating ruminal disappearance kinetics of ground forages; therefore, they are permeable to water and, theoretically, there should be no loss of insoluble forage particles as a result of applying simulated rainfall. A total of 89 bags were filled with these procedures, which included 29 bags of 1000ALF and 30 bags of both 750ALF and 500ALF.

After bags were filled with one of the three designated forage mixtures, they were sealed with an impulse sealer (Model AIE-200; American International Electric, Whittier, CA) and dried to a constant weight under forced air at 55°C. Bags were removed from the drier and immediately weighed (hot) before the forage particles could absorb water from the atmosphere. This procedure was used to obtain the most accurate estimate possible of the total amount of forage DM in each bag, without compromising subsequent analyses of fiber components by drying at temperatures >60°C (Van Soest, 1982).

Application of Simulated Rainfall

Before wetting, seven or eight bags filled with each forage mixture were assigned randomly to one of four blocks that were based on wetting cycles under a commercial rainfall simulator (Model Tlaloc 3000; Joerns, Inc., West Lafayette, IN). Within each block, one bag was assigned to remain under the rainfall simulator for either 1, 2, 4, 6, 8, or 12 h and to receive 70, 140, 280, 420, 560, or 840 mm of rainfall, respectively, which was applied at a calibrated rate of 70 mm h^{-1}. To initiate wetting procedures, 18 bags designated for Block 1 (six from each forage mixture) were placed under the rainfall simulator, and rainfall was initiated. Bags from each forage mixture were withdrawn at the time intervals described previously, allowed to drip dry on an elevated wire screen for 0.5 h, and then dried to a constant weight under forced air (55°C). Undamaged controls consisting of either one or two bags per block for each forage mixture did not receive any simulated rainfall and served as a 0-mm treatment. Although the cumulative volume of rainfall application was extremely high in comparison to typical rates of natural rainfall, much of the applied water was shed by the Dacron bags; the goal of these procedures was not to mimic field conditions but to create a gradient of DM losses similar to those observed in the field (Gordon et al., 1969; Rotz and Abrams, 1988) that could be used to validate internal markers. Despite the shedding action of the Dacron bags, all forages receiving simulated rainfall, regardless of rainfall increment, were completely saturated when they were removed from under the simulator.

After drying, bags were removed from the oven and immediately weighed as described previously to determine the final weight of forage DM contained within each individual bag. By sealing the test forages within Dacron bags, the forage DM within each bag could be determined without the need to subsample or to quantitatively transfer forages into paper bags or other containers for drying, thereby risking additional experimental error. Actual losses of DM were calculated as differences between initial and final weights of DM within each bag, and recoveries were reported as a proportion of the initial amount of DM. After evaluating Block 1, identical procedures were used to apply simulated rainfall to Blocks 2 through 4. Blank (empty) Dacron bags also were included within each block to allow for any potential correction to the weights of Dacron bags following wetting and drying; weight changes to the Dacron bags following treatment were negligible, and no corrections to the data were necessary.

Chemical Analysis of Forage

All dry forage samples were ground through a Wiley mill (Arthur H. Thomas, Philadelphia, PA) equipped with a 1-mm screen and then analyzed in duplicate for possible internal markers that included NDF, NDF with heat-stable α-amylase (NDF*a*), NDF with sodium sulfite (NDF*s*), and NDF with α-amylase and sodium sulfite (NDF*as*). Determination of NDF*as* also was followed by sequential evaluation of acid-detergent fiber (ADF*as*), hemicellulose (HEMI*as*), cellulose (CEL-L*as*), and lignin (LIG*as*). All fiber components were determined using the batch procedures outlined by ANKOM Technology Corporation (Fairport, NY). For this study, α-amylase and/or sodium sulfite were added to the NDF solution to remove or limit potential interference from starch and proteins associated with the cell wall, respectively. Similarly, ADF*as* was determined sequentially, thereby limiting potential interference from pectins during quantification of both ADF*as* and HEMI*as* (Van Soest et al., 1991).

Concentrations of NDF, NDF*a*, NDF*s*, and NDF*as* also were corrected for residual ash following combustion of insoluble residues in a muffle furnace at 500°C for 2 h. However, ash correction offered little improvement with respect to marker recovery or prediction of DM losses relative to uncorrected concentrations of these internal markers. Therefore, ash-corrected data are not reported or discussed.

Recoveries of Potential Markers

The efficacy of any internal marker is dependent on the marker being unaffected by treatment, and its complete recovery following treatment is essential. Each of the eight internal markers in this study was evaluated by quantifying the total pool of each marker on a weight basis (g), both before and after simulated rainfall treatments. Marker recoveries from forages receiving no simulated rainfall sometimes differed slightly from 1000 g kg^{-1}; these small differences reflect sampling, handling, and laboratory errors incurred during the experiment.

Calculated Dry Matter Recovery

Concentrations of internal markers before and after rainfall treatments were used to estimate recoveries of DM using an adaptation of the equation suggested by Fonnesbeck et al. (1986):

$$\text{DM recovery (g kg}^{-1}) = (CW_I/CW_R) \times 1000 \text{ g kg}^{-1}$$

where CW_I = cell wall concentration before the rainfall event and CW_R = cell wall concentration after the rainfall event.

Statistical Analysis

Initially, data for actual gravimetric recovery of DM (g kg^{-1}) were analyzed as a randomized complete block design with a 3 × 7 factorial arrangement of forage mixtures (1000ALF, 750ALF, and 500ALF) and rainfall increments (0, 70, 140, 280, 420, 560, and 840 mm). However, a strong interaction ($P = 0.002$) of forage mixture and rainfall increment was observed for this gravimetrically based response variable, which served as a standard for subsequent marker validation. Although forage mixture × rainfall increment interactions generally were not observed for concentrations and recoveries of internal markers, all data are analyzed and reported by individual forage mixture because of the strong interactions observed for losses of DM determined gravimetrically.

Within each forage mixture, all response variables were evaluated by trend analysis using the GLM procedures of SAS (1990). The sums of squares were partitioned into linear,

quadratic, and cubic effects and tested for significance with the residual error mean square. Because rainfall increments were not spaced equally, PROC IML (SAS Institute, 1990) was used to adjust coefficients for orthogonality before evaluating trends over increments of simulated rainfall.

Agreement between marker-predicted recoveries of DM and actual losses of DM determined by gravimetric procedures was tested by linear regression. Theoretically, these regressions should produce a slope of one and an intercept of zero to indicate ideal agreement between marker-predicted and gravimetric methods. Tests of homogeneity (PROC GLM) were conducted to detect differences in parameter estimates (intercept and slope) between forage mixtures. If slopes and intercepts did not differ ($P > 0.05$) across mixtures, data were combined, and a common regression equation was reported. If the regression relationships for each forage were not homogenous ($P < 0.05$), an independent regression equation was generated by the REG procedure of SAS (1990) for each forage mixture. An additional test statement was included to evaluate whether slope = 1. Throughout the study, statistical significance was declared at $P \leq 0.05$, unless otherwise noted.

RESULTS AND DISCUSSION

Determination of Actual Dry Matter Recovery

Actual losses of DM from Dacron bags ranged from 0.0 to 2.8 g DM across the three forage mixtures (data not shown). The maximum numerical loss (2.8 g DM) occurred as a result of applying 840 mm of simulated rainfall to 1000ALF; respective maximum losses from 750ALF and 500ALF were numerically less (2.4 and 2.2 g DM), but each occurred in response to the maximum rainfall increment. Overall, recoveries of DM ranged from 896 to 1001 g kg^{-1}, and declined in curvilinear patterns with cumulative rainfall amount (Fig. 1); however, these response patterns also varied with forage mixture. The highest-ordered effects for each mixture were quadratic for 1000ALF ($P < 0.001$) and 750ALF ($P < 0.008$) but cubic ($P = 0.013$) for 500ALF. Overall, recoveries of DM were generally consistent with several other studies (Scarbrough et al., 2004, 2005; Rotz et al., 1991; Fonnesbeck et al., 1986) and exhibited a range wide enough to allow a reasonable assessment of the proposed internal markers. Much greater losses of DM have been reported in some studies (Collins, 1983, 1985); however, these evaluations also included physical leaf losses, which are problematic in any study assessing rainfall effects on legumes because of the fragile nature of leaf tissues.

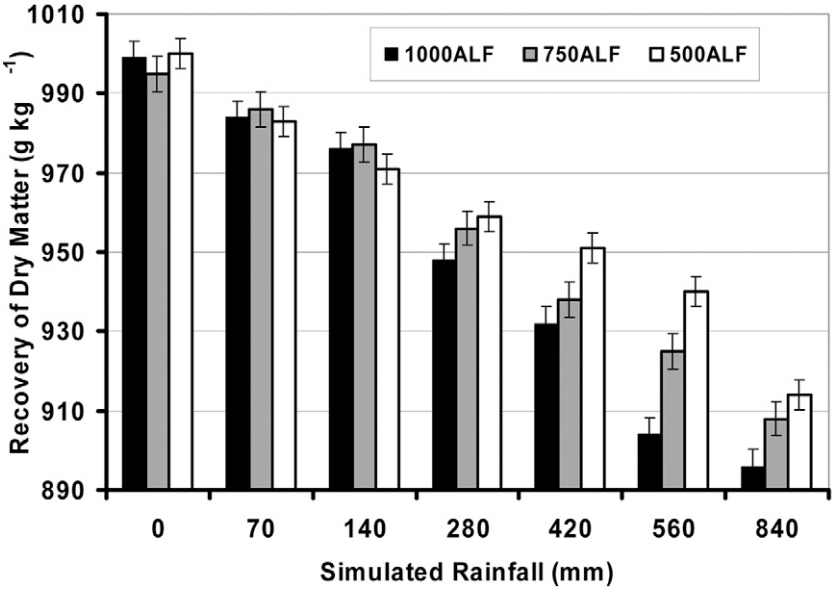

Figure 1. Actual (gravimetric) recoveries of forage dry matter (DM) from Dacron bags initially containing 26.0 ± 0.79 g DM and subjected to simulated rainfall. Highest-ordered significant effects of rainfall amount for each forage mixture were quadratic ($P < 0.001$) for 1000 g kg^{-1} alfalfa (1000ALF), quadratic ($P = 0.008$) for 750 g kg^{-1} alfalfa (750ALF), and cubic ($P = 0.013$) for 500 g kg^{-1} alfalfa (500ALF).

Concentrations of Markers

As expected, including greater proportions of orchardgrass within forage mixtures increased the concentrations of NDF, NDF*a*, NDF*s*, NDF*as*, HEMI*as*, and CELL*as* but depressed concentrations of LIG*as* (Table 1). Legumes are known to contain highly lignified cell walls but also to contain less plant cell wall than grasses of comparable digestibility (Van Soest, 1982). Before wetting, adding each increment of 250 g kg^{-1} of orchardgrass generally increased concentrations of NDF, NDF*a*, NDF*s*, and NDF*as* by about 34 g kg^{-1} but decreased concentrations of LIG*as* by about 13 g kg^{-1}. Under the conditions described for this study, concentrations of ADF*as* were largely unaffected by forage mixture.

Within each forage mixture, simulated rainfall increased concentrations of all internal markers, primarily in linear relationships with rainfall amount. This supports the premise that most cell-wall components are insoluble in water (Van Soest, 1982) and that their concentrations increase on wetting as soluble compounds are leached from the plant tissues. Previously, Collins (1991) has demonstrated that these changes within rain-soaked alfalfa forages occur primarily in response to disproportionate leaching or other losses from leaf tissues.

Marker Recovery

Within this study, marker recovery was defined as the mass of the marker recovered after wetting divided by the initial mass of the marker × 1000 g kg^{-1}; therefore, full recovery is defined as 1000 g kg^{-1}. For 1000ALF, recoveries of internal markers ranged from 901 to 1051 g kg^{-1} across all combinations of markers and rainfall increments (Table 2). For individual markers, mean recoveries averaged over all rainfall increments ranged from 988 g kg^{-1} for HEMI*as* to 1010 g kg^{-1} for LIG*as*, thereby indicating that

recoveries were essentially complete following wetting. No cubic ($P \geq 0.082$) or quadratic ($P \geq 0.163$) effects were observed for any marker. Only relatively weak linear declines with rainfall increment were observed for ADF*as* ($P = 0.049$) and CELL*as* ($P = 0.039$) but not for any other marker ($P \geq 0.256$). Similar responses were observed for 750ALF; overall mean recovery for the eight markers ranged from 1000 to 1043 g kg^{-1}, again indicating full recovery, and only HEMI*as* and LIG*as* exceeded a very narrow range of 1000 to 1020 g kg^{-1} that confined all other markers. Within the context of this relatively narrow range, the curvilinear effects of rainfall increment observed for NDF ($P = 0.042$), NDF*s* ($P = 0.035$), HEMI*as* ($P = 0.024$), and LIG*as* ($P = 0.041$) are somewhat difficult to interpret and of questionable biological significance.

For 500ALF forages, overall means for NDF, NDF*a*, NDF*s*, NDF*as*, ADF*as*, and CELL*as* ranged narrowly between 1005 and 1021 g kg^{-1}, again suggesting full recovery. Within this group of markers, recovery increased linearly ($P = 0.031$) over rainfall increments for NDF but no other effects ($P \geq 0.064$) were observed. In contrast, the overall recovery of HEMI*as* (1050 g kg^{-1}) was numerically greater than observed for other markers and increased over rainfall increments, with both linear ($P = 0.010$) and quadratic ($P = 0.013$) effects.

All internal marker techniques are dependent on complete recovery of the marker following treatment. This requirement for internal markers has been identified by Cochran and Galyean (1994) as a critical aspect in the measurement of fecal output or digestibility in livestock with internal dietary markers. Each of the internal markers evaluated in this study are found within both leaf and stem tissues; therefore, complete recovery of all shattered leaves is an essential component of their use. As a research tool, this requirement may complicate the use of internal markers in controlled experiments with legumes and may essentially eliminate their use in production-scale field studies in which shattered leaves can't be recovered quantitatively. For smaller controlled studies, such as those using wire

Table 1. Concentrations of internal markers within alfalfa (*Medicago sativa* L.)–orchardgrass (*Dactylis glomerata* L.) mixtures following wetting under a rainfall simulator.

Mixture†	Rainfall	NDF‡	NDF*a*	NDF*s*	NDF*as*	ADF*as*	HEMI*as*	CELL*as*	LIG*as*
g kg^{-1} alfalfa	mm	g kg^{-1}							
1000	0	451	453	433	431	324	107	256	73
	70	461	461	438	438	332	105	262	73
	140	482	477	460	454	348	106	271	79
	280	471	472	448	448	346	102	268	77
	420	491	490	470	461	341	120	268	79
	560	510	509	485	484	364	120	282	85
	840	492	493	474	466	346	121	271	80
	SEM	9.4	11.5	9.0	8.9	7.4	3.9	6.4	2.0
Contrasts§		*P* > *F*							
Linear		0.001	0.003	<0.001	0.001	0.017	0.001	0.053	0.001
Quadratic		0.038	0.102	0.081	0.062	0.038	0.858	0.128	0.042
Cubic		0.459	0.463	0.414	0.327	0.966	0.048	0.835	0.427
750	0	488	485	468	466	323	144	264	59
	70	498	500	486	482	328	154	268	61
	140	518	508	493	493	338	155	275	64
	280	528	523	510	507	346	161	279	68
	420	519	516	498	497	336	161	273	64
	560	537	536	518	517	348	169	283	66
	840	560	546	536	527	363	164	296	66
	SEM	7.9	7.0	7.3	7.6	5.9	3.4	4.8	1.6
Contrasts		*P* > *F*							
Linear		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.007
Quadratic		0.611	0.234	0.474	0.214	0.972	0.010	0.593	0.054
Cubic		0.083	0.317	0.094	0.192	0.115	0.855	0.180	0.066
500	0	523	515	504	499	321	178	271	48
	70	542	531	519	519	329	191	279	49
	140	542	537	528	521	330	191	283	46
	280	561	553	540	537	339	198	290	50
	420	567	554	545	541	339	202	289	51
	560	571	564	548	548	346	202	295	50
	840	592	578	566	560	353	207	305	52
	SEM	5.2	4.5	6.2	5.0	4.5	2.5	4.6	1.5
Contrasts		*P* > *F*							
Linear		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.021
Quadratic		0.116	0.034	0.146	0.025	0.369	0.004	0.548	0.942
Cubic		0.115	0.108	0.103	0.147	0.616	0.058	0.288	0.766

†Mixtures contained 1000, 750, or 500 g kg^{-1} alfalfa with the balance in orchardgrass (0, 250, or 500 g kg^{-1}, respectively).

‡NDF, neutral-detergent fiber without additives; NDF*a*, neutral-detergent fiber with heat-stable α-amylase; NDF*s*, neutral-detergent fiber with sodium sulfite; NDF*as*, neutral-detergent fiber with heat-stable α-amylase and sodium sulfite; ADF*as*, acid-detergent fiber determined sequentially following initial extraction in neutral detergent containing both α-amylase and sodium sulfite; CELL*as*, cellulose determined sequentially following initial extraction in neutral detergent containing both α-amylase and sodium sulfite; HEMI*as*, hemicellulose determined sequentially following initial extraction in neutral detergent containing both α-amylase and sodium sulfite; LIG*as*, acid-detergent lignin determined sequentially following initial extraction in neutral detergent containing both α-amylase and sodium sulfite.

§Linear, quadratic, and cubic effects of simulated rainfall amount.

Table 2. Recoveries of internal markers from alfalfa (*Medicago sativa* L.)–orchardgrass (*Dactylis glomerata* L.) mixtures following wetting under a rainfall simulator.

Mixture†	Rainfall	NDF‡	NDF*a*	NDF*s*	NDF*as*	ADF*as*	HEMI*as*	CELL*as*	LIG*as*
g kg^{-1} alfalfa	mm	g kg^{-1}							
1000	0	998	999	999	998	998	998	998	994
	70	1005	1001	995	999	1009	969	1007	977
	140	1042	1028	1036	1028	1048	969	1032	1061
	280	990	989	980	985	1012	901	993	999
	420	1014	1009	1011	996	980	1045	975	1004
	560	1022	1015	1012	1015	1016	1012	996	1051
	840	978	974	979	968	955	1008	947	982
	Overall Mean	1007	1002	1002	999	1003	988	992	1010
	SEM	18.9	24.3	20.2	19.7	21.0	35.5	22.7	26.4
Contrasts§		*P* > *F*							
Linear		0.357	0.449	0.463	0.256	0.049	0.258	0.039	0.979
Quadratic		0.217	0.391	0.412	0.364	0.198	0.757	0.485	0.163
Cubic		0.816	0.749	0.765	0.639	0.675	0.082	0.830	0.733
750	0	994	996	995	996	994	993	995	999
	70	1007	1016	1022	1020	1001	1055	998	1023
	140	1037	1022	1029	1032	1021	1049	1016	1059
	280	1034	1030	1042	1041	1025	1068	1011	1099
	420	998	997	998	1000	976	1045	968	1017
	560	1018	1022	1023	1026	995	1087	990	1027
	840	1042	1022	1039	1026	1020	1032	1019	1022
	Overall Mean	1017	1014	1020	1019	1004	1043	1000	1033
	SEM	14.8	12.3	13.0	13.5	15.4	21.0	15.4	25.2
Contrasts		*P* > *F*							
Linear		0.139	0.390	0.191	0.472	0.776	0.321	0.816	0.872
Quadratic		0.851	0.688	0.991	0.520	0.518	0.024	0.198	0.137
Cubic		0.042	0.170	0.035	0.083	0.051	0.670	0.096	0.041
500	0	1001	1002	1002	1001	1001	1001	1003	999
	70	1018	1014	1013	1023	1006	1054	1013	1009
	140	1008	1012	1017	1013	998	1042	1016	932
	280	1029	1030	1027	1032	1012	1066	1024	992
	420	1030	1023	1027	1031	1004	1080	1012	998
	560	1025	1030	1021	1032	1013	1065	1024	984
	840	1034	1026	1026	1026	1004	1064	1028	992
	Overall Mean	1019	1018	1018	1021	1005	1050	1016	988
	SEM	10.3	9.9	13.6	11.7	15.4	14.1	17.6	30.3
Contrasts		*P* > *F*							
Linear		0.031	0.064	0.232	0.152	0.761	0.010	0.331	0.870
Quadratic		0.343	0.195	0.387	0.145	0.680	0.013	0.834	0.754
Cubic		0.560	0.693	0.501	0.705	0.855	0.212	0.680	0.470

†Mixtures contained 1000, 750, or 500 g kg^{-1} alfalfa with the balance in orchardgrass (0, 250, or 500 g kg^{-1}, respectively).

‡NDF, neutral-detergent fiber without additives; NDF*a*, neutral-detergent fiber with heat-stable α-amylase; NDF*s*, neutral-detergent fiber with sodium sulfite; NDF*as*, neutral-detergent fiber with heat-stable α-amylase and sodium sulfite; ADF*as*, acid-detergent fiber determined sequentially following initial extraction in neutral detergent containing both α-amylase and sodium sulfite; CELL*as*, cellulose determined sequentially following initial extraction in neutral detergent containing both α-amylase and sodium sulfite; HEMI*as*, hemicellulose determined sequentially following initial extraction in neutral detergent containing both α-amylase and sodium sulfite; LIG*as*, acid-detergent lignin determined sequentially following initial extraction in neutral detergent containing both α-amylase and sodium sulfite.

§Linear, quadratic, and cubic effects of simulated rainfall amount.

screens or trays to contain forages while treatments are applied (Scarbrough et al., 2005; Rotz et al., 1991; Collins, 1983, 1985), internal markers should be useful for quantifying leaching losses from legumes provided that shattered leaf particles are quantitatively recovered and then adequately represented within the analyzed sample. In contrast, physical leaf losses will likely need to be determined independently, probably by direct gravimetric measure of shattered leaf particles retained on the wire screen or tray (Collins, 1983). This issue is much less important in experiments with grasses because leaves are much less fragile and less prone to shatter on contact with rain droplets.

Marker-Predicted vs. Gravimetric Recoveries of Dry Matter

Linear regressions of predicted recoveries of DM determined indirectly from concentrations of internal markers on gravimetrically determined recoveries of DM were tested for homogeneity across the three forage mixtures (data not shown). For each of the eight internal markers evaluated, slopes did not differ ($P \geq 0.067$) across forage mixtures. Among markers, only CELL*as* displayed a tendency ($P = 0.067$) for heterogeneity of slopes, while all others did not approach significance ($P \geq 0.226$). In contrast, the homogeneity of intercepts across forage mixtures varied widely with each individual marker. Neither NDF ($P = 0.276$) nor ADF*as* ($P = 0.989$) exhibited evidence of heterogeneity; however, NDF*a* ($P = 0.051$), NDF*s* ($P = 0.077$), CELL*as* ($P = 0.061$), and LIG*as* ($P = 0.053$) all exhibited tendencies for intercepts to differ, while intercepts for NDF*as* ($P = 0.028$) and HEMI*as* ($P = 0.004$) differed significantly across forage mixtures.

Despite the heterogeneous intercepts (NDF*as*) or tendencies for intercepts to differ (NDF*a* and NDF*s*) across forage mixtures, the various measures of NDF proved consistently to be the most desirable marker approach. For NDF (Fig. 2A), NDF*a* (Fig. 2B), and NDF*s* (Fig. 2C), slopes ($n = 21$) ranged from 1.00 to

1.05 and did not differ from one in any case ($P \geq 0.701$). Similarly, intercepts ranged across these markers from −57 to −12 g kg^{-1}, which did not approach ($P \geq 0.617$) statistical difference from zero. Generally, these responses are consistent with those required for a new or alternative procedure, in which the slope and intercept relating the new and standard techniques should not differ from one and zero, respectively. Simple linear relationships also explained relatively large proportions of the total variation within the data; coefficients of determination (r^2) were relatively high, ranging narrowly from 0.790 to 0.833 over these three markers. Although regression equations for the three forage mixtures differed for the NDF*as* marker (Fig. 3), many of the characteristics for individual regressions ($n = 7$) for 1000ALF, 750ALF, and 500ALF were consistent with those described for the other NDF-based markers. Specifically, slopes did not differ ($P \geq 0.103$) from one, nor did intercepts differ ($P \geq 0.083$) from zero, and r^2 statistics were relatively high, ranging from 0.775 to 0.951.

Of the four markers requiring at least two digestion steps, ADF*as* (Fig. 4A) appeared to be the most desirable, exhibiting acceptable estimates of slope (0.845) and intercept (145 g kg^{-1}) that did not differ from one ($P = 0.222$) or zero ($P = 0.231$), respectively. These were coupled with an r^2 statistic (0.714) that was reasonably competitive with NDF-based markers. It remains unclear how ADF*as* would perform as a marker if its concentration were determined directly, without the preliminary digestion in neutral detergent. Legume cell walls are known to contain significant concentrations of pectin that is insoluble in acid detergent; however, pectin is removed during the preliminary digestion in neutral detergent when ADF is determined sequentially (Van Soest, 1982).

Other markers that require multiple digestion and/or analytical steps for quantification were less desirable for several reasons. First, indices of nutritive value, such as HEMI*as*, CELL*as*, and LIG*as*, are reported inconsistently within forage-related research; therefore, their use may require additional analyses beyond those deemed most important by the researcher. In contrast, NDF (with or without heat-stable α-amylase or sodium sulfite) would likely be reported in any experiment with objectives related to forage nutritive value. Second, additional digestion and associated-weighing steps increase the probability of laboratory errors, which are then carried over into calculations of DM recovery. Within this context, regressions evaluating CELL*as*, LIG*as*, and HEMI*as* as internal markers exhibited a variety of characteristics that were less desirable than those exhibited by NDF-based markers. Specifically, r^2 statistics for CELL*as* (Fig. 4B) and LIG*as* (Fig. 4C) were somewhat poorer (0.653 and 0.524, respectively) than those observed for NDF-based markers, and they likely reflect their more tedious procedures for quantification. In addition, use of LIG*as* is further complicated by the relatively low concentrations observed in this study (≤85 g kg^{-1}; Table 1) and generally within most forages. Depressed r^2 statistics occurred for LIG*as* despite very desirable estimates of slope (1.06) and intercept (−68 g kg^{-1}) that did not differ from one ($P = 0.785$) or zero ($P = 0.764$), respectively. For HEMI*as*

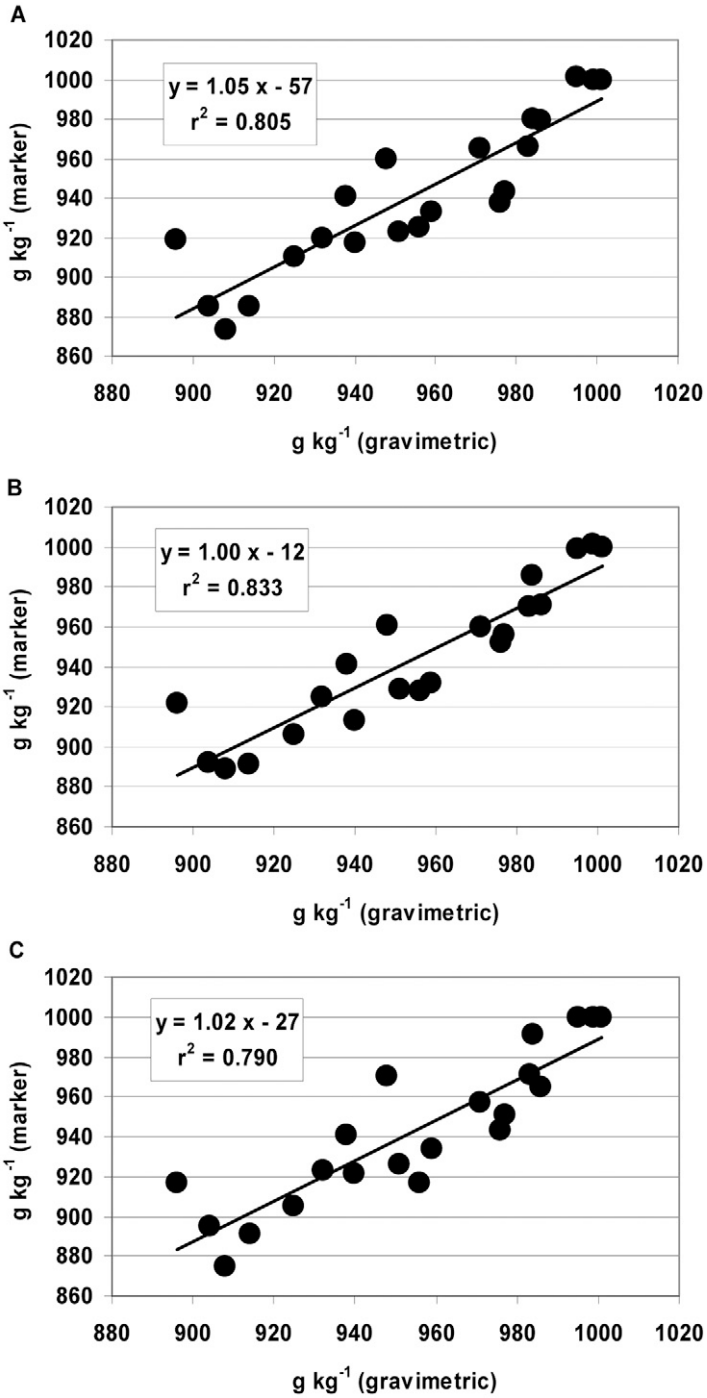

Figure 2. Linear regressions of recoveries of dry matter determined on the basis of concentrations of (A) neutral-detergent fiber without additives (NDF), (B) neutral-detergent fiber with heat-stable α-amylase (NDF*a*), and (C) neutral-detergent fiber with sodium sulfite (NDF*s*) on those determined by gravimetric techniques. Intercepts ($P \geq 0.051$) and slopes ($P \geq 0.226$) did not differ across forage mixtures (1000 g kg^{-1} alfalfa, 750 g kg^{-1} alfalfa, and 500 g kg^{-1} alfalfa); therefore, data are combined ($n = 21$). In each case, the combined slope ($P \geq 0.701$) and intercept ($P \geq 0.617$) did not differ from one and zero, respectively.

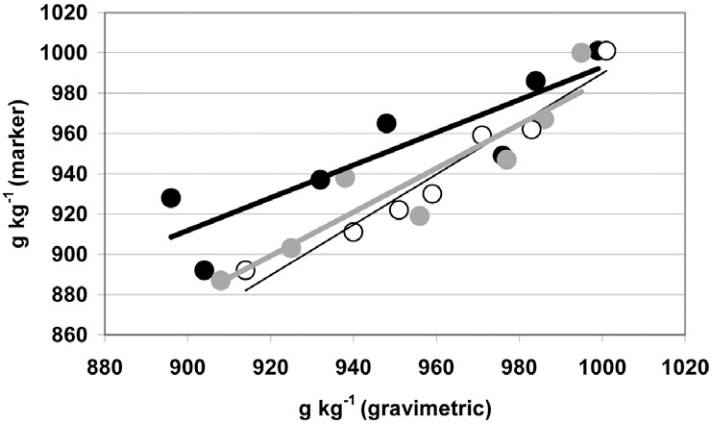

Figure 3. Linear regression of recoveries of dry matter calculated from concentrations of neutral-detergent fiber determined with heat-stable α-amylase and sodium sulfite (NDF*as*) on those obtained by gravimetric techniques. Slopes for 1000 g kg^{-1} alfalfa (1000ALF), 750 g kg^{-1} alfalfa (750ALF), and 500 g kg^{-1} alfalfa (500ALF) were homogenous (P = 0.259), but intercepts were not (P = 0.028); therefore, data are presented by forage mixture (n = 7). Regression equations for 1000ALF (black circles, bold black line; $y = 0.81x + 182$, $r^2 = 0.775$; $P = 0.009$), 750ALF (gray circles, bold gray line; $y = 1.09x - 101$, $r^2 = 0.851$; $P = 0.003$), and 500ALF (white circles, light black line; $y = 1.25x - 263$, $r^2 = 0.951$; $P < 0.001$) all exhibited slopes ($P \geq 0.103$) and intercepts ($P \geq 0.083$) that did not differ from one and zero, respectively.

(Fig. 5), estimates of slopes and intercepts for 1000ALF, 750ALF, and 500ALF did not differ statistically from one ($P \geq 0.076$) or zero ($P \geq 0.060$), respectively; however, the magnitudes of slopes (1.27 to 1.56) and intercepts (−297 to −587 g kg^{-1}) that were observed across forage mixtures suggests a potential for bias that might be demonstrated with a more extensive data set.

CONCLUSIONS

Cell-wall components appear to be suitable internal markers for DM loss or recovery from rain-damaged alfalfa or alfalfa–grass mixtures. The use of internal markers represents a significant improvement over the simple gravimetric methods used previously, which have been quite problematic. All internal markers were essentially recovered completely from alfalfa and alfalfa–orchardgrass mixtures following wetting. Therefore, relationships between predicted and actual recoveries of DM were good ($r^2 \geq 0.524$) for all the internal markers evaluated. Neutral-detergent fiber, measured either with or without heat-stable α-amylase or sodium sulfite, was most effective (r^2 = 0.775 to 0.951). Neutral-detergent fiber appears to be the most suitable marker for experimental use because (i) it comprises a greater proportion of the total DM pool than any other fiber component; (ii) procedures for quantification are relatively rapid, inexpensive, and do not require multiple analytical or digestion steps; (iii) relationships between predicted and actual recoveries of DM were generally superior to those obtained with other markers; and (iv) NDF is determined as part of most routine forage-testing packages. Internal

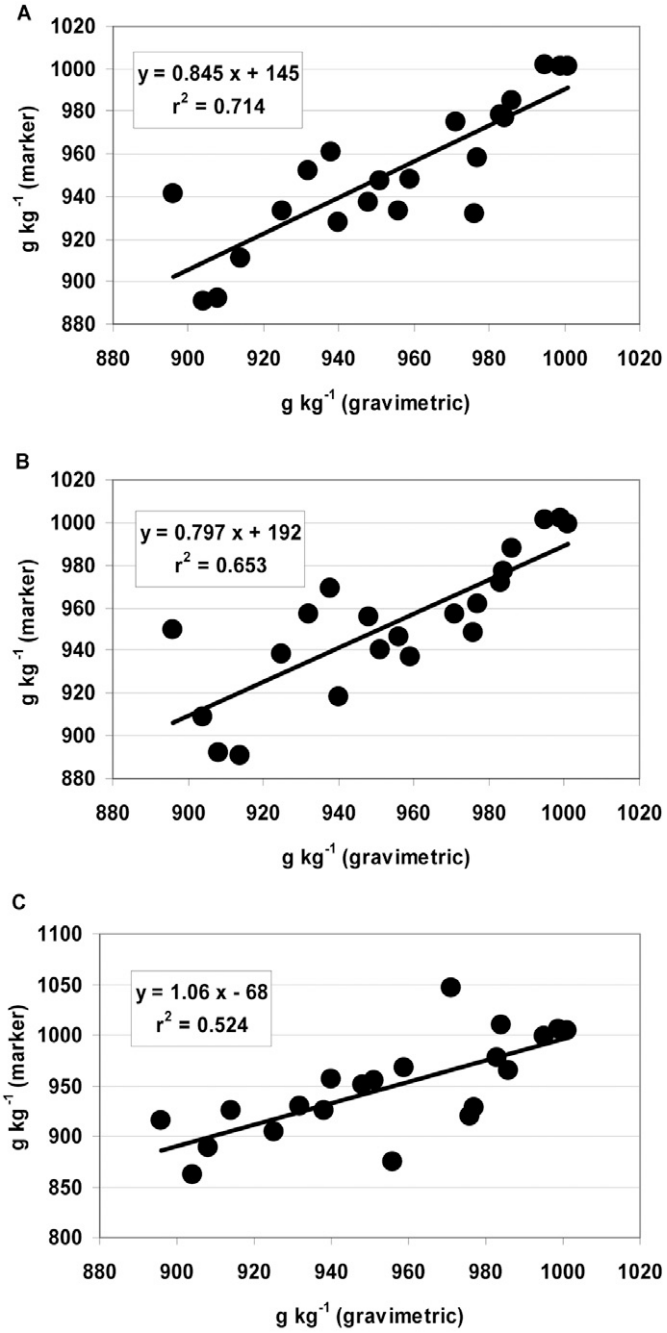

Figure 4. Linear regressions of recoveries of dry matter calculated from concentrations of (A) acid-detergent fiber determined sequentially following initial extraction in neutral detergent containing both α-amylase and sodium sulfite (ADF*as*), (B) cellulose determined sequentially following initial extraction in neutral detergent containing both α-amylase and sodium sulfite (CELL*as*), and (C) acid-detergent lignin determined sequentially following initial extraction in neutral detergent containing both α-amylase and sodium sulfite (LIG*as*) on those obtained by gravimetric techniques. Intercepts ($P \geq 0.053$) and slopes ($P \geq 0.067$) did not differ across forage mixtures [1000 g kg^{-1} alfalfa (1000ALF), 750 g kg^{-1} alfalfa (750ALF), and 500 g kg^{-1} alfalfa (500ALF)]; therefore, data are combined (n = 21). In each case, the combined slope ($P \geq 0.144$) and intercept ($P \geq 0.147$) did not differ from one and zero, respectively.

markers requiring multiple digestion steps are probably less acceptable, largely because their quantification procedures are more tedious, and with the exception of ADF*as*, they are analyzed less consistently in forage research projects.

Generally, the proposed methodology appears suitable for measuring primarily leaching losses, but it is contingent on complete recovery of all shattered leaf particles before conducting postwetting laboratory analyses. Considering this limitation, the most appropriate use of this technique may be in closely controlled experiments in which wilting legumes are positioned on screens or trays and then subjected to some type of simulated rainfall. These types of studies easily can be designed so shattered leaf particles can be recovered quantitatively. In this context, losses of DM can be reasonably estimated from pre- and postwetting concentrations of NDF. However, use of the technique as a field research or management tool is limited by the need to recover shattered leaf particles quantitatively. For field or production-scale situations, reasonable estimation of losses of DM determined from representative forage samples collected pre- and postwetting will have to be verified experimentally and can only be accomplished if leaf shatter is negligible.

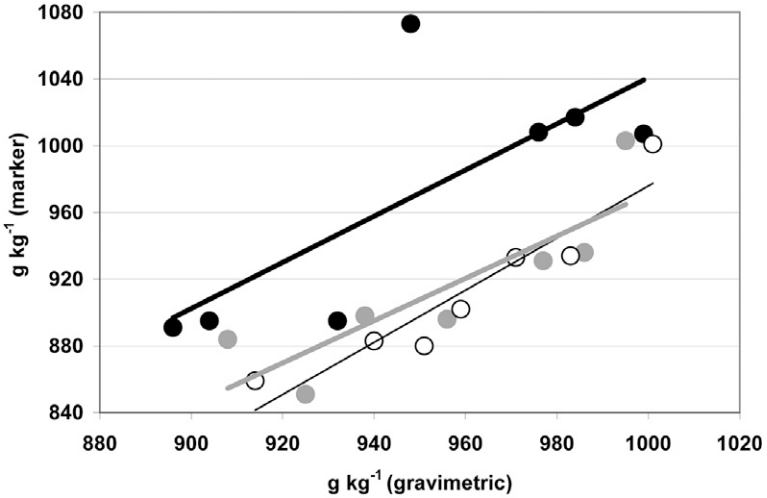

Figure 5. Linear regression of recoveries of dry matter calculated from concentrations of hemicellulose determined sequentially following initial extraction in neutral detergent containing both α-amylase and sodium sulfite (HEM*las*) on those obtained by gravimetric techniques. Slopes for 1000 g kg^{-1} alfalfa (1000ALF), 750 g kg^{-1} alfalfa (750ALF), and 500 g kg^{-1} alfalfa (500ALF) were homogenous (P = 0.914), but intercepts were not (P = 0.004); therefore, data are presented by forage mixture (n = 7). Regression equations for 1000ALF (black circles, bold black line; $y = 1.38x - 343$, $r^2 = 0.554$; $P = 0.055$), 750ALF (gray circles, bold gray line; $y = 1.27x - 297$, $r^2 = 0.732$; $P = 0.014$), and 500ALF (white circles, light black line; $y = 1.56x - 587$, $r^2 = 0.885$; $P = 0.002$) exhibited slopes ($P \geq 0.076$) and intercepts ($P \geq 0.060$) that did not differ from one and zero, respectively.

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